Fawaz, S., Martin Alonso, A., Qiu, Y., Ramnath, R., Stowell-Connolly, H., Gamez, M., May, C., Down, C., Coward, R. J., Butler, M. J., Welsh, G. I., Satchell, S. C., & Foster, R. R. (2024). Adiponectin Reduces Glomerular Endothelial Glycocalyx Disruption and Restores Glomerular Barrier Function in a Mouse Model of Type 2 Diabetes. *Diabetes*, *73*(6), 964-976. https://doi.org/10.2337/db23-0455

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10.2337/db23-0455

Adiponectin reduces glomerular endothelial glycocalyx disruption and restores glomerular barrier function in a mouse model of type 2 diabetes.

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Running title: Adiponectin and glomerular endothelial glycocalyx restoration in diabetes

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Word count: (4000) **4726**

Number of figures: 5

Number of tables: 2

Abstract: (200)

Adiponectin has vascular anti-inflammatory and protective effects. Whilst adiponectin is known to protect against the development of albuminuria, historically the focus has been on podocyte protection within the glomerular filtration barrier (GFB). The first barrier to albumin in the GFB is the endothelial glycocalyx (eGlx), a surface gel-like barrier covering glomerular endothelial cells (GEnC). In diabetes, eGlx dysfunction occurs before podocyte damage, hence we hypothesized that adiponectin could protect from eGlx damage to prevent early vascular damage in diabetic kidney disease (DKD). Globular adiponectin (gAd) activated AMPK signalling in human GEnC through AdipoR1. It significantly reduced eGlx shedding and the TNFα-mediated increase in syndecan-4 (SDC4) and MMP2 mRNA expression in GEnC in vitro. It protected against increased TNFα mRNA expression in glomeruli isolated from db/db mice, and genes associated with glycocalyx shedding (SDC4, MMP2 and MMP9). In addition, gAd protected against increased glomerular albumin permeability (Ps'alb) in glomeruli isolated from db/db mice, when administered to mice (i.p) and when applied directly to glomeruli (ex vivo). Ps'alb was inversely correlated with eGlx depth in vivo. In summary, adiponectin restored eGlx depth, which was correlated with improved glomerular barrier function, in diabetes.

Article highlights

a. Why did we undertake this study?
    - Adiponectin protects against diabetic kidney disease.
    - Understanding its role on glomerular endothelial barrier properties would help formulate a strategic therapeutic approach.
b. What is the specific question we wanted to answer?
    - Can adiponectin protect against glomerular eGlx disruption to restore glomerular albumin permeability?
c. What did we find?
    - Adiponectin protects against eGlx shedding and restores albumin permeability, in experimental diabetes.
d. What are the implications of our findings?
    - Activation of the GEnC adiponectin signalling pathway may be an early treatment strategy for diabetic patients at risk of DKD.

Introduction:

The over-secretion of pro-inflammatory adipokines, such as TNF-α, combined with hyposecretion of anti-inflammatory adipokines, such as adiponectin and leptin, is thought to be a major mechanism involved in lifestyle-related diseases, including type 2 diabetes, hypertension and atherosclerosis. Adiponectin plasma levels are reduced in obese [1], and type 2 diabetic humans [2] and mice. Adiponectin is a hormone secreted primarily by adipocytes and over the last two decades it has been identified as an insulin-sensitizer, an anti-inflammatory and anti-diabetic adipokine [3, 4]. Adiponectin is considered favourable for cardiac and vascular health, alleviating oxidative stress in endothelial cells [5]. Its main mode of action is through its receptors, AdipoR1 and AdipoR2, but can also bind to T-Cadherin (expressed in smooth muscle cells, pericytes and endothelial cells). This acts as a third AdipoR, leading to accumulation in tissues [6], stimulating biogenesis of intracellular exosomes and enhancement of ceraminidase, thereby reducing cellular ceramide production [7]. AdipoR1 is highly expressed in skeletal muscle and has high affinity for the globular domain of adiponectin (gAd) while AdipoR2 is highly expressed in the liver and has intermediate affinity for gAd [8]. AdipoR1 is expressed in renal cells, including glomerular endothelial cells (GEnC), mesangial cells, epithelial cells, proximal tubular cells, and podocytes whereas AdipoR2 is expressed in all kidney cell types, but to a lesser extent than AdipoR1 [9]. AdipoR1 signals through AMP-activated protein kinase (AMPK) and has anti-inflammatory, antifibrotic and antioxidant effects in the kidney [10, 11]. Moreover, adiponectin has been implicated in exerting beneficial renal effects against the development and progression of albuminuria in diabetic and non-diabetic renal diseases [12-14]. Interestingly, adiponectin-deficient mice are albuminuric in the absence of diabetes, and both albuminuria and podocyte AMPK signalling is restored by adiponectin [13]. This demonstrates that adiponectin is important in glomerular physiology.

Dysregulation of the adiponectin system occurs in disease conditions, such as chronic kidney disease, type 1 and type 2 diabetes, with or without nephropathy [15-17], obesity and cardiovascular disease [18]. Low plasma adiponectin levels are associated with endothelial dysfunction and vascular injury [19] and are inversely related with albuminuria in non-diabetic obese individuals [13]. They are also associated with an increased risk of adverse cardiovascular events in patients with end-stage renal failure [20].

Diabetes causes disruption to all glomerular components, but GEnC and glycocalyx dysfunction occurs early, in the microalbuminuric phase of the disease [21]. Under normal conditions the glomerular filtration barrier, which consists of fenestrated GEnC covered by a surface glycocalyx, glomerular basement membrane and podocytes, prevents the passage of albumin into the urine [22]. The eGlx is a heterogeneous structure consisting of core proteins such as SDCs or glypicans, decorated with glycosaminoglycan (GAG) side chains forming a hydrated poly-anionic gel [23]. In vitro studies have shown that human GEnC express several cell surface proteoglycans, including SDCs, glypicans, and biglycan [24]. Animal studies have shown that almost all SDCs (1 to 4) are expressed in the kidney, however, SDC1 and SDC4 are prominent in the glomerulus [24].

The degradation of the eGlx has been implicated in the pathogenesis of endothelial dysfunction and (micro)albuminuria in diabetes and [25] occurs earlier than podocyte dysfunction [26]. EGlx can be damaged by factors, such as TNF-α [24] and salt and aldosterone [27]. Protecting the eGlx is an exciting novel approach to prevent the early development of albuminuria and hence diabetic kidney disease (DKD). Some agents which can protect against damage to the eGlx in GEnC (whether in culture or in db/db animal models) have been identified [28, 29]. We aimed to determine whether adiponectin can protect the glomerular eGlx against damage in response to diabetic milieu and hence restore the albumin permeability barrier.

Materials and Methods:

Human Kidneys

Human kidney tissue for this study was obtained, with ethical approval, from kidneys retrieved but not suitable for transplantation (n=4, 3 males, 1 female). Chunks of cortex were flash frozen for mRNA extraction and glomeruli were isolated using graded sieves in a laminar flow hood (MSC/BIO -Envair 89 Rossendale Lancs UK). Kidneys were kept on ice during the whole sieving process and glomeruli were collected from the 125µm pore sieves and either mRNA or protein was extracted and stored at -80 °C for future treatments.

Mouse glomeruli

Isolated glomeruli were obtained by passing mouse renal cortex sequentially through graded sieves and collected from the 75µm pore sieve. These were either mRNA or protein extracted and stored at -80°C for future treatments.

Cell Culture

The CiGEnC used in this study have been previously extensively characterised [30] (RRID:CVCL_W185) and are regularly tested for mycoplasma. Briefly, primary GEnC were transduced with a temperature sensitive simian virus 40 large tumour antigen (SV40LT) construct and human telomerase (hTERT). This allows immature CiGEnC to proliferate at temperature of 33°C and then become quiescent and fully differentiate at 37°C, a non-permissive temperature for 3-5 days. Monolayers of CiGEnC are cultured in EBM-2MV media which contains basal medium (Scientific Laboratory Supplies (SLS), Nottingham, UK,) supplemented with 5% foetal bovine serum (FBS) and the EGM2-MV bullet kit (SLS) in the absence of supplied vascular endothelial growth factor and gentamicin.

Treatments

CiGEnC or isolated glomeruli (human and mouse) were treated with gAd (PeproTech, London, UK) at an optimum concentration of 2.5µg/ml [13] for varying times, as indicated. TNF-α, used to model pro-inflammatory aspects of the diabetic milieu, was used at 10ng/ml, as previously described [24].

Generation of adiponectin receptor knockdown cells

The lentiviral vectors containing the shRNA of human AdipoR1 (Clone ID: VGH5518-200170736 - V2LHS_134726, Cat number: VGH5526- EG51094) or a scrambled non-target sequence (RHS4348) were purchased (Dharmacon Horizon Discovery, Cambridge, UK). Briefly, CiGEnC at 40-60% confluency was incubated with the lentiviral particle in the presence of polybrene at 1:100 ratios ($1*10^6$TU/ml final concentration) for 4 h in serum free media. The infected cells were cultured then in complete media for 48 h, followed by a puromycin selection at 0.8µg/ml for 3 consecutive days to obtain a stable knockdown cell line. The knockdown efficiency was confirmed by qPCR and Western blotting.

RNA isolation and Quantitative RT-PCR (qPCR)

RNA was extracted from CiGEnC or isolated human and mouse glomeruli using Qiagen RNeasy Kit (Qiagen, Manchester, UK. RNA was quantified (Nano drop, Thermo-Fisher Scientific, Waltham, USA) and converted to cDNA using a high-capacity RNA to cDNA kit (Thermo-Fisher Scientific #4387406). Real-time PCR was performed using a StepOnePlus Real-Time PCR System (Thermo-Fisher Scientific). Primer specificity and optimal concentration was ensured prior to any quantitative reaction to a qPCR using Fast SYBR Green master mix (Thermo-Fisher Scientific #4385612). The primers used are detailed in Table 1. All primers were validated using serial dilutions of cDNA, with a goodness of fit R^2 minimum

value of 0.99. All qPCRs were performed in triplicate. Human β-actin or GAPDH primers were used as housekeeping genes. Data were presented normalised to the housekeeping gene ($2^{-\Delta CT}$) or normalised to the housekeeping gene and then control sample ($2^{-\Delta\Delta CT}$).

Western Blot

Protein was extracted from cells using cold radio immunoprecipitation assay buffer (RIPA) (Thermo-Fisher Scientific #89901). For tissues and glomeruli, a more robust tissue lysis buffer was used (Bio Basic Inc, Markham, Canada #BSP006). All samples were stored at - 80°C, until required. Total protein lysates were blotted on PVDF membranes and then incubated with primary antibody (Table 2) at 4°C overnight. Incubation with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody at 1:10,000 dilutions was then performed for 1 h at room temperature on an orbital shaker. Immunoreactive bands were visualised using Clarity ECL Western Blotting Substrate (Bio-Rad, Hemel Hempstead, UK) on a AI600 imager (GE Healthcare, Amersham, UK) and quantified using ImageJ (NIH, https://imagej.nih.gov/ij/).

Shed sulphated GAG

An Alcian blue colourimetric assay was used to quantify the amount of sulphated GAG shed into the cultured media from the surface of CiGEnC, as previously [31]. Cells were serum starved for 2 h and then treated as indicated. The media was harvested, centrifuged at 800g for 3 min and added to a freshly prepared solution of 0.4% Alcian blue in 0.5M sodium acetate, 30mM magnesium chloride hexahydrate and 2.8% of sulphuric acid (pH~2). Absorbance at 490nm was measured after 15 min incubation. The linear relation between GAG mass and decreased absorption of 490nm by Alcian blue solution was used to quantify supernatant GAG content, referenced to known concentrations (0 to 500µg/ml) of chondroitin sulphate standards.

ELISA

After 2 h of TNF-α and/or gAd treatment, conditioned medium was collected and normalised to total protein concentration using bicinchoninic acid reagent assay kit (Thermo-Fisher Scientific #23227). Cellular levels of SDC4 were quantified using a SDC4 ELISA (R&D Systems DY2918), as per the manufacturer's instructions.

Animal work

Experiments were performed in accordance with the guide for the care and use of laboratory animals, Eighth edition (2011). All animal procedures performed conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and with approval of the University of Bristol local ethics committee and the British Home Office (Project License: P855B71B4). Animals were kept in a specific pathogen free facility, and housed in a conventional cage, typically in groups of 4-6, with enrichment. They had a 12 h light/dark cycle, with free access to food and water.

Animal model of diabetes

Male leptin-receptor mutant BKS.Cg-+Leprdb/Leprdb/OlaHsd, Harlan, UK mice (db/db, RRID:IMSR_ENV:HSD-H174) were used as a model of insulin-resistant type 2 diabetes. Age-matched lean mice (BKS.Cg-m+/+Leprdb/OlaHsd) were used as control mice. Hyperglycaemia was apparent at 7 weeks of age (lean 10.13 ± 0.68 vs db/db 23.87 ± 2.2 mmol/L, SEM $p<0.0001$) and db/db mice were significantly heavier (27.23 ± 0.63 vs db/db 21.23 ± 0.33 SEM, $p<0.0001$) as previously published [32]. Mice were kept on highly absorbent bedding, which was changed daily. Mice were monitored for reduced mobility and reduced grooming but remained visibly well and no adverse effects were recorded. At 12 weeks of age, the mice were culled, and glomeruli were isolated for protein/mRNA extraction (n=5 each group). An additional cohort of mice (n=6 for each group; lean, db/db and db/db + gAd) was used to quantify glomerular albumin permeability and eGlx depth. The primary output for

gAd treatment on db/db mice was glomerular albumin permeability, not uACR, due to the difficulties of ensuring an empty bladder before treatment and collection of urine 2 h later. Db/db mice were randomly assigned to groups. The lean and db/db groups were i.p. injected, outside of the home cage room in the morning, with vehicle (saline solution) or a single dose of gAd (PeproTech) of 2.5µg/ml in 300µl vehicle (giving an approximate circulating dose of 0.195µl/ml), for 2 h prior to a cardiac perfusion. In a further cohort, db/db mice were given glycosaminoglycan (GAG) enzymes following gAd administration to confirm that the protective effect of gAd is negated. Briefly, 12-14 week-old db/db male mice were given gAd (n=12) or vehicle (saline, n=12) by i.p. injection 2 h before cardiac perfusion. After 1.5 h from the time of i.p. injection, a subgroup of each received chondroitinase (0.087 mU/g; cat. C3667, Sigma) and Hyaluronidase (15 mU/g; cat. 385931, Sigma) via tail vein under anaesthesia (n=6 each group). Mice were maintained under anaesthesia at 37°C for 30 min before cardiac perfusion.

Glomerular Permeability Assay (P's alb)

Glomerular albumin permeability (Ps'alb) was quantified to measure changes directly and sensitively to the glomerular filtration barrier [33]. Briefly, mouse glomeruli were isolated from mice perfused with 4% bovine serum albumin (BSA) in Ringer (Ringer-BSA). Glomeruli were incubated in 36.5 mg/ml Octadecyl rhodamine B chloride (R18, Thermo-Fisher Scientific) for 15 min, then washed in 4% Ringer-BSA, followed by 15 min incubation in 30mg/ml Alexa Fluor 488-BSA (Thermo-Fisher Scientific). Glomeruli from db/db mice were treated for 30 min in the presence of 2.5µg/ml gAd, AF488-BSA at 37°C or left untreated. An individual glomerulus was trapped on a custom-made petri dish and the perfusate was switched from 30 mg/ml labelled 488- BSA to 30mg/ml unlabelled BSA. A Nikon Ti-E inverted confocal microscope (Nikon Instruments Inc., Melville, NY) was used to capture the fluorescence

intensity. The rate of decline in fluorescence intensity within the loop of the capillaries for the first minute was used to calculate Ps'alb as previously described.

Lectin staining

Paraffin-embedded kidney sections (5µm) were used for lectin staining as previously described [34]. Briefly, after blocking the sections with blocking buffer, the sections were incubated with biotinylated Maackia Amurensis Lectin-1 (MAL1, 2mg/ml) 1:100, pH 6.8, overnight at 4^{o}C. After 5 washes, the sections were incubated with streptavidin AF488 (1:500, Thermo-Fisher Scientific), pH 6.8, for 1 h at room temperature. The nuclei were then counterstained with 40, 6-diamidino-2-phenylindole (DAPI) (Thermo-Fisher Scientific) and the cell membrane labelled with R18 (1:1000) were incubated for 10 min. The sections were examined using an AF600 LX wide-field fluorescence microscope (Leica Microsystems, Milton Keynes, UK) and images captured.

Endothelial glycocalyx depth: fluorescence profile peak-to-peak analysis

Imaging analysis was used as a measure of eGlx depth on lectin-stained tissue sections, as previously [34]. Briefly, a line was drawn across the glomerular capillary loop, perpendicular to the cell membrane. Fluorescence intensity profiles were then generated for MAL1-AF488 and R18. The distance between the peak signal from the MAL1-488 (inside the capillary loop and R18 label) and the R18 fluorescent profiles is an index of glycocalyx depth (peak-to-peak) [34]. Lectin based peak-to-peak analysis has been validated against quantitative EM [35, 36]. Glycocalyx depth was determined using a blinded automated methodology, as published previously [35]. Briefly, we developed an ImageJ macro to take multiple measurements in a pre-selected capillary loop and generate fluorescence intensity profiles for the lectin components of the GEnGlx and endothelial cell label. Gaussian curves were applied to the raw intensity data of each plot for peak-to-peak measurements (dashed lines in Figure 5Bii). The

mean was subsequently determined from 200 lines per capillary loop, from 4 capillary loops/glomerulus, 4 glomeruli in a mouse, and n=6 mice in each group.

Statistical Analysis:

Graphs and statistics were made using GraphPad Prism (Version 8.4.0, RRID:SCR_002798) and p values of less than 0.05 were considered significant. Data are presented as mean ± SEM. For comparing differences between two groups, unpaired t-test with Welch's corrections was performed. Whilst comparing over two parametric data sets, either one-way or two-way analysis of variance (ANOVA) was performed with post-hoc Bonferroni's analysis. If data were not normally distributed, a Kruskal-Wallis Test was performed. Experiments were performed independently at least 3 times (n>3) with each repeated in triplicate for qPCR and Western blot results. The relationship between eGlx depth and glomerular albumin permeability was correlated using Pearson's correlation coefficient, giving a value between -1 and +1 with 0 meaning no correlation and 1 meaning complete positive correlation.

Data and resource availability:

The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request. Resources also available on request.

Results:

Human GEnC express adiponectin receptors (AdipoR) and respond to adiponectin in vitro.

We confirmed that AdipoR1 and AdipoR2 are expressed in CiGEnC (**Figure 1A** and **B**) at the protein and mRNA levels and that AdipoR1 was more highly expressed at the mRNA and protein level across kidney tissues and cells. In order to understand how gAd exerts its effects on CiGEnC, initially both a dose response and time course of gAd treatment was carried out (**Supp Figure 1**). The physiological level of circulating gAd is 2.5µg/ml [37], and similarly, the optimum concentration yielding a sustained phosphorylation of AMPK in CiGEnC was 2.5µg/ml. The maximum signalling effect of gAd on AMPK phosphorylation was 30 min. CiGEnC were treated with gAd for 30 min and the phosphorylation of known adiponectin regulated signalling effectors was investigated. There was significant phosphorylation of AMPKα, ACC, Akt and p-38 at 30 min (**Figure 1C** and **D)**.

AdipoR1 is important for adiponectin-mediated signalling in GEnC.

AdipoR1 has previously been shown to be important in glomerular signalling. To confirm that AdipoR1 is important in adiponectin signalling in CiGEnC, a knockdown cell line of AdipoR1 was generated using shRNA. Knockdown of AdipoR1 mRNA and protein was confirmed (**Figure 2A, C-D**), without impacting on AdipoR2 mRNA expression (**Figure B**). Following this, CiGEnC were treated with gAd for 30 min. AMPK is considered a predominant signalling pathway for adiponectin through AdipoR1, upstream of ACC, in normal physiology and is downregulated in diabetes [38]. Increased AMPKα and ACC phosphorylation in response to gAd treatment is shown in the scrambled controls as predicted (**Figure 2 E-G,** 2.1-fold increase). However, AMPKα (**Figure 2F**) and ACC phosphorylation (**Figure 2G**) was

abolished in the AdipoR1 knockdown CiGEnC. This demonstrates the importance of AdipoR1 in mediating adiponectin signalling in GEnC.

Adiponectin ameliorates TNFα-induced glycocalyx shedding.

The protective effect of gAd treatment on the expression of different components of the GEnC glycocalyx was examined, with or without stimulation with TNF-α. There was a significant increase in SDC4 mRNA in response to TNF-α treatment (3.2-fold) after 2 h, which was significantly reduced in the presence of gAd (2.0-fold) (**Figure 3A**). Of note, gAd treatment alone did not alter SDC4 mRNA expression. SDC4 protein shedding into conditioned medium by TNF-α was also significantly decreased by gAd (**Figure 3B)**. Similarly, the increase in sulfated GAG in the conditioned medium of TNF-α stimulated cells was significantly decreased by gAd, suggesting that adiponectin can ameliorate the impact of TNF-α on GAG shedding in CiGEnC (**Figure 3C**).

We have previously shown that TNF-α treatment of CiGEnC significantly upregulated the levels of both MMP2 and MMP9. Here, we show that the TNF-α-induced MMP2 upregulation was attenuated by gAd in CiGEnC (**Figure 3D),** but not MMP9 **(Figure 3E).** MMP2 was successfully knocked down at the mRNA and protein level using shRNA lentivirus in CiGEnC (**Figure 3F-G**). The increase in SDC4 mRNA expression in response to TNF-α was reduced in MMP2 knockdown CiGEnC compared to scrambled controls (1.9-fold) (**Figure 3H),** compared to a 3.2 fold-increase previously (**Figure 3A**). In the MMP knockdown cells, gAd did not reduce SDC4 mRNA. Taken together these results suggest that the TNF-α induced increase in SDC4 mRNA is partially dependent upon MMP2 and that the effect of gAd to reduce the TNF-α induced increase in SDC4 mRNA in control cells is through blocking MMP2 (since no effect was seen in the MMP2 knockdown cells).

Adiponectin signals to glomeruli directly and prevents the mRNA upregulation of glycocalyx-related genes in db/db glomeruli.

To confirm the physiological relevance of the abovementioned results, glomeruli were isolated from human and mouse kidneys and stimulated with gAd for varying times. The optimum dose of gAd in mouse glomeruli was determined first (**Supp Figure 2**) which peaked at 2.5µg/ml. gAd induced AMPK phosphorylation within 30 min in human and mouse glomeruli, confirming that gAd acts directly on glomeruli in both species (**Figure 4A-B**). Furthermore, in a diabetic animal model, glomeruli from 12-week-old lean and db/db mice were isolated, treated with gAd or vehicle and mRNA was extracted. In the vehicle treated db/db glomeruli there was an increase in TNF-α, SDC4, MMP2 and MMP9 mRNA (**Figure 4C-F**). However, in the gAd treated db/db glomeruli, all but MMP9 mRNA were significantly reduced.

Adiponectin protection from increased albumin permeability in diabetes is correlated with restored glomerular eGlx depth

To understand whether adiponectin could restore glomerular albumin permeability and protect from eGlx damage, 12-week-old db/db mice were given a single i.p injection of gAd, 2 h prior to perfusion, or left untreated and compared to lean littermate control mice (n= 6 each group). Glomerular albumin permeability (Ps'alb) was quantified in isolated glomeruli. There was a 2.5-fold increase in Ps'alb in diabetic mice compared to lean mice, which was significantly reduced by gAd (lean: 2.65±0.16; db/db: 8.25 ±0.5; db/db +gAd i.p.: 3.12±0.17, **Figure 5Ai**). Db/db glomeruli were also treated with gAd ex vivo and demonstrated a significant reduction in Ps'alb, although it was not completely normalised (db/db + gAd ex vivo: 5.8±0.25 cm/sec x 10^{-7}, **Figure 5Aii**).

MAL-1 is a lectin that binds to GAG within the eGlx and can be measured on the luminal surface of the labelled GEnC membrane. The fluorescence profile peaks of R18 (red; labelling

cell membranes) and MAL1 (green; labelling GAG in the eGlx) were compared and the distance between the two was used as a measure of eGlx depth (**Figure 5B**). There was a 1.5-fold reduction in the eGlx in diabetic glomeruli, which was significantly restored by gAd (lean: 224±11.7; db/db: 98.3±12.3; db/db + gAd i.p.: 164.14±7.0 nm **Figure 5Bii**). Importantly, these measures of glycocalyx depth were very similar to previous measures, imaged in live and unfixed mesenteric rat vessels [36] suggesting limited impact of fixation on depth. Correlation analysis was carried out between eGlx depth and Ps'alb, demonstrating a significant inverse relationship (**Figure 5C**).

Vascular GAG disruption prevents adiponectin protection from increased albumin permeability in diabetes.

We have previously shown that an i.v. bolus of chondroitinase and hyaluronidase (which are restricted to the vasculature due to their high molecular weight [39]), given 30 min before sacrifice, significantly increases glomerular albumin permeability and significantly reduces eGlx depth, without effecting podocyte glycocalyx [29, 33]. We created 4 groups of mice (n=6 in each group); db/db mice ± adiponectin 2 h before sacrifice and then gave ± enzyme bolus in the final 30 min. The expectation was that gAd treatment followed by enzyme treatment would negate the protective effect of gAd on glomerular albumin permeability.

Three animals were excluded due to either being identified as outliers using Graphpad Prism (n=2) or due to an equipment failure (n=1). There was a significant 1.3-fold decrease in Ps'alb in diabetic mice given vehicle compared to diabetic mice given gAd (db/db plus vehicle: 6.66 ±0.18; db/db +gAd i.p.: 5.1±0.12, **Supplementary Figure 3)**. The Ps'alb for diabetic mice with vehicle was lower than previously for diabetic mice (**figure 5A**). We believe this is due to the extended isoflurane anaesthesia before sacrifice that is known to act as a vasodilator and antihypertensive. In addition, gas anaesthetics, including isoflurane, have been shown to

protect eGlx [40]. GAG enzymes did not further increase Ps'alb in diabetic mice, as anticipated since eGlx has already been compromised (**Figure 5Biii**), and the effect of gAd was negated when GAG enzymes were subsequently delivered to disrupt the eGlx (no significant difference to diabetes plus vehicle).

Discussion:

We have shown, for the first time, that adiponectin can protect against TNF-α mediated eGlx shedding in GEnC and restores glomerular eGlx depth in diabetes. EGlx depth was inversely correlated with glomerular albumin permeability, suggesting causation, in line with our previous studies [34].

Importantly, this work provides a novel mechanism to enhance barrier function via glomerular eGlx in DKD. The eGlx is the primary barrier to protein in the glomerular filtration barrier and is damaged in diabetes before the development of kidney disease. Thus, this novel mechanism of adiponectin-induced signalling, highlighting restoration of eGlx, suggests earlier therapeutic intervention can stop progression of DKD and associated financial and health burdens.

Our results complement previous studies that have demonstrated that adiponectin protects from albuminuria [12-14]. It is well established that adiponectin can signal through podocytes and that adiponectin-deficient mice are albuminuric and have podocyte foot process effacement, causing (macro)albuminuria, which can be restored by adiponectin [13]. An orally active AdipoR agonist, AdipoRon, was shown to protect from kidney disease associated with obesity, endotoxins and diabetic nephropathy, including upregulation of AMPK and phosphorylation of ACC in GEnC [41, 42]. GEnC ultrastructural changes were not quantified in these in vivo studies by electron microscopy (yet podocyte and glomerular basement membrane changes were) [42]. In diabetic nephropathy, AdipoRon reduced glomerular lipotoxicity and oxidative stress in podocytes and endothelial cells, resulting in reduced albuminuria and glomerulosclerosis when given from 16-20 wk of age [42]. It was also shown to be protective in experimental adiponectin resistance [43]. Our study does not detract from previous results, rather it adds temporal information around microvascular damage and mechanism. We demonstrate that adiponectin can signal directly to GEnC, to rapidly induce normalisation in

albumin permeability in 12 wk old db/db mice, via restoration of eGlx depth, independently of podocyte Glx changes, which is a novel mechanism. We have previously seen rapid eGlx restoration linked with restored albumin permeability by angipoietin-1 in type 1 diabetic rats [33]. Thus, adiponectin has powerful implications in preventing the progression of *micro*albuminuria, an *early* microvascular dysfunction event in DKD that precedes podocyte dysfunction, which is related to macroalbuminuria. Patients with diabetes may therefore benefit from adiponectin - targeted therapy, in the early stages of DKD.

SDC1 and SDC4 are the most prominent syndecans in the glomerulus and we have previously shown that SDC4 was most highly expressed in CiGEnC and freshly isolated human GEnC [24]. We have also shown that SDC4 shedding could be induced by the inflammatory mediator, TNF-α, through an MMP2/9-dependent mechanism [24], consistent with previous publications [44]. TNF-α has been shown to be dysregulated and linked to type 2 diabetes [45, 46]. Previously, we have shown that TNF-α-induced SDC4 shedding is associated with increased SDC4, MMP2 and MMP9 mRNA expression and that these are all upregulated in diabetic glomeruli and associated with glomerular eGlx damage [24, 34].

Of note, the increase in SDC4 mRNA expression associated with increased SDC4 protein shedding, suggested that SDC4 mRNA upregulation could be used as a proxy for TNF-α-mediated SDC4 protein shedding, as in Figure 3H. Moreover, previously, treatment with an MMP inhibitor, Batimastat, reduced this increase in SDC4 mRNA (~2 fold) [24], which supports our results whereby MMP2 knockdown reduced the increase in SDC4 mRNA 1.9-fold.

Intervention with Batimastat restored glomerular eGlx depth and albumin permeability [34]. In this manuscript, we show that adiponectin can protect against a TNF-α-induced increase in SDC4 and MMP2 mRNA in cultured GEnC and in diabetic glomeruli. MMP2 knock-down in

GEnC prevented the protective effect of adiponectin on TNF-α induced increases in SDC4. In addition, adiponectin given both i.p (2 h before sacrifice), and directly to ex vivo glomeruli (30 min at 37°C), significantly reduced glomerular albumin permeability. This indicates that adiponectin acts directly on glomerular cells to restore barrier properties. Treatment ex vivo did not return albumin permeability to baseline, as it did in vivo, which suggests that either 30 min was not as effective as 2 h, or that adiponectin exerts additional systemic effects. In addition, the restoration of Ps'alb by adiponectin in vivo was negated when vascular glycocalyx was disrupted. In this study, we demonstrate for the first time that adiponectin can protect against eGlx shedding by TNF-α in vitro and in vivo. We provide in vitro evidence that we can target adiponectin signalling in GEnC.

As described previously, low plasma adiponectin levels are associated with endothelial dysfunction and vascular injury [19]. Low plasma adiponectin levels are also inversely related with albuminuria in non-diabetic obese individuals [13], suggesting a relationship between loss of glomerular filtration integrity and low adiponectin levels. Rather confusingly, low adiponectin levels have also been associated with better physical health in those with chronic kidney disease [47]. This is in contradiction to other studies, where adiponectin was implicated in exerting beneficial renal effects against the development and progression of albuminuria in diabetic and non-diabetic renal diseases [12-14]. The recently coined "adiponectin paradox" describes high circulating adiponectin levels associated with both cardiovascular risk and cardiovascular health. A Mendelian randomisation study suggested that high adiponectin serum levels contribute to cardiovascular mortality [48]. However, this has since been contradicted by another Mendelian randomisation study [49]. Instead, raised serum adiponectin is suggested to be associated with high mortality risk in those with *existing* cardiovascular disease. A potential resolution of the paradox is provided by evidence that high levels of adiponectin are due to adiponectin resistance that develops in response to chronic cardiovascular disease,

potentially through reduced sequestering by T-Cadherin [50]. Hence adiponectin signalling through AdipoR (independent of circulating adiponectin) remains a viable therapeutic target.

Of note, AdipoRon, which activates Adiponectin receptors directly, has been shown to be protective in experimental adiponectin resistance [43]. Of significance, low circulating adiponectin has been shown to have a causal effect on reduced estimated glomerular filtration rate (eGFR), with higher adiponectin levels being protective [51]. Pre-clinical data for adiponectin receptor agonists is strong in many diseases and human trial plans are in progress (e.g Allysta Pharmaceuticals plans to initiate human trials against dry eye disease using the AdipoR agonist, ADP355, as eye drops)[52]. Our results suggest that adiponectin targeted therapeutics may be a viable mechanism that should be explored for clinical vascular protection in DKD and early prevention of disease.

In summary, adiponectin targeted signalling in GEnC has therapeutic potential for protecting against early glomerular eGlx dysfunction in DKD.

Acknowledgements.

We would like to acknowledge the Wolfson Bioimaging Facilities for providing an excellent service in confocal training and use of confocal microscopes.

Authorship contribution

SF co-ordinated and helped design the project, interpret data, wrote the manuscript, and approved the final version. AMA designed and conducted significant animal experiments, interpreted the data, edited the manuscript and approved the final version. YQ contributed to data collection, helped to interpret data, edited the manuscript and approved the final draft. RR contributed to data collection, helped to interpret data, edited the manuscript and approved the final draft. HSC and MG also helped to conduct significant animal experiments, edited the

manuscript and approved the final version. CM contributed to data collection, helped to interpret data, edited the manuscript and approved the final draft. CD advised on lectin image analysis, helped to interpret data, edited the manuscript and approved the final draft. RJC edited the manuscript and approved the final draft. MJB contributed to experimental design and edited the draft. GIW helped design the project, helped to interpret data, edited the manuscript and approved the final version. SCS conceived and designed the project, helped to interpret data, edited the manuscript and approved the final version. RRF helped designed and manage the project, helped to interpret data, edited the manuscript and wrote the final version.

Guarantor Data Access and Responsibility Statement

Dr. Rebecca Foster is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Funding

SF has received funding from Innovative Medicines Initiative 2 Joint Undertaking of Biomarker Enterprise to Attack Diabetic Kidney Disease (BEAt-DKD) consortium. AMA was funded by Kidney Research UK project grant RP_005_20221128 and Elizabeth Blackwell Institute MRC Confidence in Concepts award. YQ was funded by British Heart Foundation project grants PG/16/35/32139 and PG/20/7/34849). RR was funded by Diabetes UK project grant 18/0005795 and Kidney Research UK project grant KS_RP_002_20190917. HSC was funded by a Wellcome Trust Partnership Award Fellowship 2023. MG was funded by British Heart Foundation project grant PG/20/10187. CM was funded by Diabetes UK project grant 19/0006037. CD was funded by David Telling Charitable Trust. RJC and SCS are members of the Biomarker Enterprise to Attack Diabetic Kidney Disease (BEAt-DKD) consortium. This project has received funding from the Innovative Medicines Initiative 2 Joint Undertaking

under grant agreement No 115974. The JU receives support from the European Union's Horizon 2020 research and innovation programme and EFPIA and JDRF. Any dissemination of results reflects only the author's view; the JU is not responsible for any use that may be made of the information it contains. MJB was funded by a Kidney research UK development grant.

Prior presentation

Parts of this study were presented in abstract form at 67th British Microcirculation Society, 3-4th April, 2017 and at European Diabetic Nephropathy Study Group 32nd Annual Meeting, 25th May, 2019.

Duality of interest

No potential conflicts of interest relevant to this article were reported.

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Figure legends

Figure 1: Human GEnC express adiponectin receptors and respond to adiponectin *in vitro* A: Representative Western blots of AdipoR1 and AdipoR2 demonstrating kidney lysates with adipocytes as a positive control, with β-actin as a loading control. **Aii**: Densitometry of n=4 Western blots showing AdipoR1 and |AdipoR2 protein level normalised to β-actin. **B**: qPCR analysis graph representing the relative mRNA expression of AdipoR1 and AdipoR2 across kidney tissue, glomerular cells and adipocytes (n=4 repeats each in triplicate). Data are plotted as the mean $2^{-(\Delta CT)}$ of each triplicate with mean. **Ci:** Representative Western blot demonstrating gAd (at 2.5µg/ml) or vehicle effects at 30 min in CiGEnC on AMPK, ACC, p-38 and Akt phosphorylation (duplicates are shown). **Cii:** Densitometry confirmed the phosphorylation of AMPK, ACC, p-38 and Akt in response to gAd at 30 min compared to controls. Densitometry was performed on 3 representative blots from 3 independent repeats (n=3) showing levels of phosphorylated protein of interest normalised to β-actin loading control, bars/dots represent means ±SEM, one-way ANOVA *p<0.05, ** p <0.01, *post hoc* analysis (Bonferroni).

Figure 2: **AdipoR1 is important for adiponectin-mediated signalling in GEnC.** AdipoR1 knockdown was carried out using shRNA. **A**. Reduced AdipoR1 mRNA expression (gene of interest/housekeeping gene; $2^{-(\Delta CT)}$ was confirmed by 3 separate shRNA constructs in GEnC, n=4 One-way ANOVA *p<0.05, **p<0.01. **B**. AdipoR2 mRNA expression (gene of interest/housekeeping gene; $2^{-(\Delta CT)}$) was not significantly affected. **C.** Representative Western blot demonstrating the knockdown extent of AdipoR1 in shRNA v726 **D**. Densitometry confirmed the knockdown of AdipoR1 protein expression in CiGEnC, data normalised to β-actin loading control, scatter dot represent means ±SEM, n= 3, unpaired t test, * p<0.05. **E:** Representative Western blot demonstrating the effects of gAd (2.5µg/ml) on scrambled control, and AdiporR1 knockdown (R1 KD) on AMPK and ACC phosphorylation in CiGEnC. **F and G:** Densitometry showing the extent of phosphorylation of AMPK and ACC in the 3 cell clones after 30 min of gAd stimulation. Data normalised to β-actin loading control, dots represent means ±SEM, n= 4, one-way ANOVA, ** p < 0.01, ***p<0.001, **** p<0.0001. *post hoc* analysis (Bonferroni).

Figure 3: Adiponectin ameliorates TNFα-induced glycocalyx shedding. GEnC were treated with or without 10ng/ml TNF-α for 2h, in the presence/absence of 2.5µg/ml gAd. **A**: qPCR analysis of SDC4 mRNA levels, normalised to GAPDH are shown. Data are plotted as the mean $2^{-(\Delta CT)}$ (gene of interest/ housekeeping gene) of each triplicate with mean. **B**: SDC4 protein expression is quantified by ELISA in the media of treated cells. **C**: Sulphated glycosaminoglycans were quantified in the media of treated cells by Alcian blue colourimetric assay. MMP2 (**D**) and MMP9 (**E**) mRNA levels in CiGEnC treated with TNF-α (10ng/ml) and/or gAd (2.5µg/ml). **A** to **E:** n=5 one-way ANOVA, *p<0.05, ** p <0.01, ***p<0.001, **** p<0.0001. *post hoc* analysis (Bonferroni). MMP2 was knocked down using 3 shRNA different sequences in GEnC (v33, v47, v49) or not, using scrambled control. (**F**) qPCR data analysis highlighting the decreased expression of MMP2 mRNA in CiGEnC by all 3 different shRNA sequences. Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. GAPDH used as the housekeeping gene control, n=4 one-way ANOVA, **** p <0.0001 *post hoc* analysis (Bonferroni). (**Gi**) Representative Western Blot demonstrating the protein knockdown of MMP2 by shRNA clone v47. (**Gii**) Densitometry showed significant knockdown of MMP2 protein expression by clone v47. Data normalised to β-actin loading control, dots represent mean ±SEM, one-way ANOVA, *** p < 0.001. *post hoc* analysis (Bonferroni). **H**. SDC4 mRNA levels in MMP2 knockdown CiGEnC treated with TNF-α and/or gAd. Data are plotted

as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. GAPDH used as the housekeeping gene control, n=5 one-way ANOVA, *** $p < 0.001$ *post hoc* analysis (Bonferroni).

Figure 4: Adiponectin signals to glomeruli directly and prevents the mRNA upregulation of glycocalyx-related genes in db/db glomeruli. Human (**A**) and mouse (**B**) glomeruli were isolated and treated ex vivo with 2.5μg/ml gAd. Representative Western Blots demonstrating gAd effects at 30 min in human (**Ai**) and mouse (**Bi**) ex vivo glomeruli on AMPK phosphorylation. **Aii** and **Bii.** Densitometry confirmed significant phosphorylation of AMPK in response to gAd after 30 min in human and mouse glomeruli. Densitometry was performed on blots from independent repeats (n=4) showing levels of protein of interest normalised to β-actin loading control, dots represent means ±SEM, one-way ANOVA, * $p < 0.05$, **$p<0.01$, post hoc analysis (Bonferroni). **C-F**: qPCR analysis graph representing the mRNA expression of TNF (**C**), SDC4 (**D**), MMP2 (**E**) and MMP9 (**F**) in ex-vivo (wild type or db/db) sieved glomeruli treated with or without adiponectin. Data are plotted as the mean $2\text{-}(^{\Delta\Delta CT})$ of each triplicate with mean, n=5 one-way ANOVA, *$p<0.05$, **$p<0.01$ ****$p<0.0001$ post hoc analysis (Bonferroni).

Figure 5: Adiponectin protection from increased albumin permeability in diabetes is correlated with restored glomerular eGlx depth. Twelve wk old lean, db/db and db/db i.p. injected with gAd for 2 hours, were culled. One kidney was paraffin embedded and the other was used to isolate glomeruli. Isolated glomeruli from the db/db groups were further treated with vehicle or gAd for 30 min (db/db). (**A**) Isolated glomeruli were then incubated with R18 followed by Alexa Fluor 488–bovine serum albumin and Ps'alb quantified. **(Ai)** Total number of glomeruli; 18 from lean (wt/wt), db/db and db/db +gAd for the 2h gAd i.p. injection and **(Aii)** 15 glomeruli for db/db (vehicle) and (db/db gAd 30 min). Ps'alb was quantified and statistical analysis was carried out on n=6 mice. Data are expressed as mean ± SEM. *** $P<0.001$, **** $p<0.0001$. One-way ANOVA (**B**) Kidney tissue sections were stained with R18, to label cell membranes red, and the glycocalyx binding lectin, MAL-1 (green). Confocal imaging at high power allowed fluorescent profiles to be plotted and the distance between peaks (peak to peak) to be quantified (a measure of eGlx depth). (**Bi**) Representative images show glomerular capillaries labelled red (R18) and the luminal glomerular endothelial glycocalyx labelled green with MAL-1. White arrowheads highlight capillary loops. (**Bii**) A linear region of interest is drawn across a single capillary loop (white line) and the fluorescent profile of R18 (red) and MAL-1 (green) are shown. A gaussian distribution is applied to the profiles (dotted lines) and the distance between the two peaks is quantified, as a measurer of eGlx depth. (**Biii**). The distance between R18 and MAL-1 fluorescence is summarised. Individual glomeruli are shown, and statistical analysis was applied to n=5 mice per group. **C**: Correlation between Ps'alb and eGlx depth using Pearson's correlation analysis.

Supplementary figure 1: Phosphorylation of p-AMPK α in response to a dose and time dependent effect of gAd on CiGEnC. Representative Western Blot demonstrating the concentration dependent effect of gAd (1, 2.5, 10, and 25 μg/ml) (**A**) and time dependent effect (0.5,1,2,4 and 24hr) (**C**) in CiGEnC on AMPK-α phosphorylation respectively. Densitometry confirmed the phosphorylation of AMPK in response to gAd at optimum concentration of 2.5 μg/ml (**B**) and at a maximum time of 30 minutes (**D**). Data normalised to β-actin loading control, dots represent mean ±SEM, n= 3, one-way ANOVA, ** $p < 0.01$, *** $P<0.001$, **** $p<0.0001$. post hoc analysis (Bonferroni).

Supplementary figure 2: Phosphorylation of p-AMPK α in response of a dose dependent effect of gAd on mouse glomeruli for 30min. A. Representative Western blot demonstrating the concentration dependent effect of gAd (1, 2.5 and 5μg/ml) in mouse glomeruli on AMPK-

α phosphorylation after 30 minutes. **B.** Densitometry confirmed the phosphorylation of AMPK-α in response to gAd. Data normalised to β-actin loading control, dots represent mean±SEM, n= 3, one-way ANOVA, * $p < 0.05$. *Post hoc* analysis (Bonferroni).

Supplementary figure 3: Vascular GAG disruption prevents adiponectin protection from increased albumin permeability in diabetes. Twelve-14 wk old db/db, i.p. injected with gAd or saline vehicle for 2 hours, were then t.v. injected ± glycosaminoglycan (GAG) enzymes for 30 min before sacrifice and glomeruli isolated. Isolated glomeruli were then incubated with R18 followed by Alexa Fluor 488–bovine serum albumin and Ps'alb quantified. Total number of glomeruli; db/db saline;24, db/db +gAd; 24, db/db + GAG enzymes; 22, db/db + gAd+GAG enzymes; 28. Ps'alb was quantified, and statistical analysis was carried out on n=5, 5, 5 and 6 mice respectively. Data are expressed as mean ± SEM. Kruskal-Wallis Test, * $P<0.05$. *post hoc* analysis (Dunn's Multiple Comparison Test).

Table 1 qPCR primer sequences

Human primers	Forward primers (3”-5”)	Reverse primers (5”-3”)
β -actin	ATGTGGCCGAGGACTTTGATT	AGTGGGGTGGCTTTTAGGATG
Adiponectin	CAGGCCGTGATGGCAGAT	AGTCTCCAATCCCACACTGAAT
AdipoR1	ACAAGGTCTGGGAGGGACGT	ACAAGGTCTGGGAGGGACGT
AdipoR2	TGCAGCCATTATAGTCTCCCAG	GAATGATTCCACTCAGGCCTAG
Syndecan-4	CCTCCTAGAAGGCCGATACTT	AGGGCCGATCATGGAGTCTT
MMP2	GAGACCATGCGGAAGCCAAGATG	GGTGTGTAACCAATGATCCTGTATGT
MMP9	GCCCCAGGAGTCTGGAT AAGTTGG	GTCGAATCTCCAGACAC GCCCC
Mouse primers		
β-actin	CTGTCCCTGTATGCCTCTG	ATGTCACGCACGATTTCC
GAPDH	GTTGTCTCCTGCGACTTCA	GGTGGTCGAGGGTTTCTTA
TNF-α	CACAGAAAGCATGATCCGCGACGT	CGGCAGAGAGGAGGTTGACTTTCT
Adiponectin	GATGGCAGAGATGGCACTCC	CTTGCCAGTGCTGCCGTCAT
AdipoR1	ACGTTGGAGAGTCATCCCGTAT	TGTCCAGATGTTGCCAGTCTCTGTGTG
AdipoR2	GCCCAGCTTAGAGACACCTG	CTCTGTGTGGATGCGGAAGAT
Syndecan-4	CCCTTCCCTGAAGTGATTGA	AGTTCCTTGGGCTCTGAGG
MMP2	GCTATGTCCACTGTGGGTGGAAAT	GATCCCTTGATGTCATCATGGGATAATAGACC

Table 2 Primary and secondary antibodies

Antibody (ab) Type	**Name**	**Source**	**Molecular weight (kDa)**	**Dilution**	**Validation**	**Supplier**	**Research Resource Identifiers**
Primary ab	p-AMPKα (Thr172)	Rabbit	62	1:1000	CST	CST#2535	RRID:AB_331250
Primary ab	p-ACC (Ser79)	Rabbit	280	1:1000	CST	CST#11818	RRID:AB_2687505
Primary ab	p-Akt (Ser473) (D9E)	Rabbit	62	1:1000	CST	CST#4060	RRID:AB_2315049
Primary ab	p-P38MAPK	Rabbit	43	1:1000	CST	CST#4511	RRID:AB_2315049
Primary ab	AdipoR1 (EPR6626)	Rabbit	44	1:1000	Human kidney lysate	Abcam Ab#126611	RRID:AB_11129655
Primary ab	AdipoR2	Rabbit	43	1:1000	Human kidney lysate	Thermiofisher #36-3100	RRID:AB_2849888
Primary ab	B-actin	Mouse	42	1:1000	N/A	Sigma #A5441	RRID:AB_476744
Primary ab	MMP2		60	1:1000	Human kidney lysate	Proteintech 10373-2-AP	RRID:AB_2919317
Secondary ab	Goat anti mouse	Goat		1:500		Life technologies UK, #A11001	RRID:AB_2534069
Secondary ab	Goat anti-rabbit	Goat		1:500		Life technologies UK, #A11008	RRID:AB_143165